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STRATEGIES FOR THE EXTRACTION AND ANALYSIS OF NON-EXTRACTABLE POLYPHENOLS FROM PLANTS

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HIGHLIGHTS

- Review of extraction and analysis of non-extractable polyphenols (NEPs), 2008-2017
- Current knowledge of NEPs and their interaction with plant matrices
- Different methodologies used to carry out the extraction and purification of NEPs
- Latest methodologies developed for their separation, quantification and identification

ABSTRACT

The majority of studies based on phenolic compounds from plants are focused on the extractable fraction derived from an aqueous or aqueous-organic extraction. However, an important fraction of polyphenols is ignored due to the fact that they remain retained in the residue of extraction. They are the so-called non-extractable polyphenols (NEPs) which are high molecular weight polymeric polyphenols or individual low molecular weight phenolics associated to macromolecules. The scarce information available about NEPs shows that these compounds possess interesting biological activities. That is why the interest about the study of these compounds has been increasing in the last years. Furthermore, the extraction and characterization of NEPs are considered a challenge because the developed analytical methodologies present some limitations. Thus, the present literature review summarizes current knowledge of NEPs and the different methodologies for the extraction of these compounds, with a particular focus on hydrolysis treatments. Besides, this review provides information on the most recent developments in the purification, separation, identification and quantification of NEPs from plants.

Abbreviations: CDTA, cyclohexane diamine tetraacetic acid; COSY, correlated NMR spectroscopy; 1D, one dimensional NMR; ^{2}D, two dimensional NMR; DAD, diode array detector; DMAC, dimethylaminoacinnamaldehyde; DP, degree of polymerization; DPPH, α-diphenyl-β-picrylhydrazyl; ESI, electrospray ionization; EPP, extractable polyphenols; FLD, fluorescence detector; FRAP, ferric reducing ability of plasma; GC, gas chromatography; HBMC, heteronuclear multiple bond correlation; HHDP, hexahydroxydiphenic acid; HILIC, hydrophilic interaction liquid chromatography; HMQC, heteronuclear multiple quantum coherence; HPLC, high performance liquid chromatography; HSCCC, high-speed counter-current chromatography; HPP, hydrolysable polyphenols; HTs, hydrolysable tannins; LCxLC, multidimensional liquid chromatography; LLE, liquid-liquid extraction; MALDI, matrix-assisted laser desorption/ionization; MDDE, microwave-assisted simultaneous distillation and dual extraction; MS, mass spectrometry; NEPA, non-extractable proanthocyanidins; NEPs, non-extractable polyphenols; NOESY, nuclear overhauser effect spectroscopy; NP, normal phase; ORAC, oxygen radical absorbance capacity; PAs, proanthocyanidins; PHWE, pressurized hot water extraction; PLE, pressurized liquid extraction; PP, polymeric polyphenols; Q, quadrupole; QQQ, triple quadrupole; ROESY, rotating frame of reference; RP, reverse phase; SFE, supercritical fluid extraction; SLE, solid-liquid extraction; SPE, solid phase extraction; TOCSY, totally correlated NMR spectroscopy; TOF, time of flight; TPC, total phenolic content; UAE, ultrasound assisted extraction; UHPLC, ultra-high performance liquid chromatography; UV, ultraviolet detector.

Keywords: Non-extractable polyphenols, polymeric polyphenols, extraction, analysis, plants.

1. INTRODUCTION

In the last few years, an increased interest on phenolic compounds has arisen, mainly because their potential health effects such as antioxidant, among others [1]. Phenolic compounds have an important advantage compared with other antioxidants, since they are synthesized by plants and are widespread in plant-based foods. Therefore, they are consumed in great amounts on a daily basis. Thus, their consumption plays an important role in the prevention of several diseases associated with oxidative stress, such as cancer, cardiovascular and cognitive diseases [2,3].

Plant polyphenols are a family of hundreds of antioxidants made up of different subclasses (phenolic acids, flavonoids, stilbenes, lignans, and others). However, the studies which quantify the total phenolic content in plants underestimated the real content. The reports on the total phenolic content deal with extractable polyphenols (EPPs) derived from aqueous or aqueous-organic extraction [4]. Nevertheless, an important phenolic fraction, called non-extractable polyphenols (NEPs), is not taken into account in the analysis of total phenolic compounds because they remain in the extraction residue [4,5].

Currently, the majority of works found in the literature are relative to the study of the EPPs concentration and composition in food [6]. The scarce information available shows that NEPs possess interesting biological activities such as antioxidant [7], antiinflamatory [8], antidiabetic [9], chemopreventive [10] and anticholesterolemic [11]. Moreover, some NEPs produce a balance on the microbiota in obese people, prevent the risk of cardiovascular diseases and inhibit proteins glycation [8]. The researchers have observed that these compounds are abundant in food [4] but their extraction from the matrix as well as their qualitative and quantitative analysis are

considered a challenge because the analytical methodologies available present some limitations. They are not sensitive, selective, and specific, and there are not many commercially available standards of oligomeric and polymeric polyphenols.

This review article presents an updated critical overview on the extraction and analysis methods used to determine NEPs in plants. The goal of this review is to provide the reader with a broad view of the different classes of NEPs and what extraction, residue treatments after extraction, purification and analytical protocols and techniques are used to achieve the characterization of NEPs from plants. Especially the literature search is focused on the last ten years (2008-17) without at all being exhaustive, showing the last methods employed for the extraction and analysis of NEPs from natural samples, although there are some studies on this subject prior to 2008 [12-16]. The last part is conclusions and an outlook towards the near future in terms of extraction and analytical methods development to characterize NEPs.

2. TYPES AND DISTRIBUTION OF NEPs IN PLANTS

NEPs are high molecular weight polymeric polyphenols or individual low molecular weight phenolics associated to macromolecules, mainly polysaccharides, constituents of dietary fiber and proteins. Besides, they can be retained in the food matrix inaccessible to solvents due to different interactions with the plant matrix [6,8]. Thus, after a conventional extraction, NEPs remain in the corresponding residues [17]. Currently, NEPs with high molecular weight are the least known and studied. For this reason, these compounds are introduced in depth in this section.

NEPs are divided into two groups, condensed tannins which are also called proanthocyanidins (PAs), and their monomers are flavan-3-ols (see Figure 1); and hydrolysable tannins (HTs), deriving from gallic and ellagic acids (see Figure 2).

2.1. Condensed tannins or proanthocyanidins

PAs are polyhydroxyflavan oligomers or polymers [18]. Hydroxylation pattern in ring A and B and in the stereochemistry of C-3 produces different monomeric flavanols. The flavanol monomers are normally bound by carbon-carbon links in 4 → 6 or 4 → 8 position (PAs type B), but in some plants an additional C2 → C7 ether-linkage (PAs type A) may appear (Figure 1). Sometimes, PAs may be esterified in certain positions with gallic acid or exceptionally with sugars [19].

These compounds can be found in the plant vacuole or associated to other cellular components, such as the cell wall [20]. The association occurs mainly in tegumental tissues and outer parts of fruits and stems through different reactions between PAs and polysaccharides. Basically, PAs are in the peel of fruits, in coating of seeds, in the bran of grains and in barks [21].

2.2. Hydrolysable tannins

Hydrolysable tannins are polyesters of a sugar moiety and phenolic acids. These compounds are composed of a polyol central core (normally glucose) acylated by a variable number of gallic or ellagic acid units and their derivatives, forming intermediate to high molecular weight phenolic compounds [18]. When phenolic acid is like a monomeric unit gallic acid, the compounds are called gallotannins (Figure 2.a, 2.b) [21]. On the other hand, when the phenolic compound is hexahydroxydiphenic acid (HHDP), which forms ellagic acid when it is hydrolysed through elimination of water, the compounds are termed ellagitannins (Figure 2.c, 2.d) [21]. Ellagitannis are the products of oxidation, leading to C-C linkages between galloyl residues of glucogalloyl molecules that form HHDP units [22]. Ellagitannins are characterized by one or more HHDP units esterified to a sugar core [21]. Some authors have shown that these compounds are present in nuts [8], dark chocolate,

almonds, cashew apples [23] and plants (*Paeonia suffruticosa, Paeonia lactiflora, Schinus terebinthifolius*) [21], among others.

3. INTERACTION WITH COMPOUNDS FROM MATRIX

As mentioned above, NEPs remain retained in the plant matrix inaccessible to solvents during the extraction process [8]. In this sense, chemistry interactions between matrix compounds and NEPs promote this retention. Principally, interactions or associations depend on the characteristics of the cell wall and NEPs [24].

The associations of these compounds to the cell wall are influenced by the molecular weight or the percentage of galloylation of the phenolic molecule [24]. In fact, phenolic compounds with higher mean degree of polymerization (DP) and percentage of galloylation tend to remain attached to the cell wall [24, 25]. For instance, Fournand et al. [26] observed that the extractable PAs fraction from grape skins had a lower DP and less galloylated subunits than non-extractable PAs (NEPAs) fraction.

Additionally, the chemical composition and porosity of the cell wall can have influence on the aggregation with polysaccharides and NEPs [27]. The size of pores of the cell wall may differ between 4 to 10 nm of diameter. In this way, a little diameter of these pores restricts the interactions of high molecular weight polyphenols with polysaccharides due to the impossibility of penetration of phenolic compounds into the cell wall. So, high molecular weight polyphenols are not retained in the cell wall [28].

In general, the compounds which can interact with polyphenols, have carboxyl groups of uronic acids (hemicellulose and pectin) and hydroxyl groups of pectins [29]. Thus, high pectin levels favor the affinity of polyphenols with the cell wall [27].

NEPs are also associated to polysaccharides of the cell wall through hydrogen bonds between the hydroxyl groups of polyphenols and the oxygen atoms of the cross-

linking ether bonds of sugars [27]. In addition, phenolic acids, such as hydroxycinnamic and hydroxybenzoic acids, can be associated to lignin through ether linkages with hydroxyl groups from the aromatic ring, and structural carbohydrates and proteins via ester linkages with carboxylic groups [30]. Moreover, some polysaccharides have the ability to develop secondary structures which originate hydrophobic regions able to encapsulate NEPs through hydrophobic interactions [24, 27]. Besides, these hydrophobic interactions can be facilitated by the increase of ionic strength. In contrast, an increase of temperature reduces the interaction between polysaccharides and polyphenols [24].

In addition, different researchers have shown that an increase in the proportion of catechin units might improve the formation of hydrogen bonds and hydrophobic interactions between proteins and polyphenols. It is due to the flexibility conformation of the polymer as a result of the formation of a kink [24].

On the other hand, the oxidation of vegetables leads to the production of polyphenol derivatives. These derivatives can react with the NH_2 group of lysine and CH_2S group of methionine of enzymes and other proteins to form complexes. In this way, the covalent interactions phenolic compounds-proteins may decrease the extraction of NEPs [31, 32].

The interactions between NEPs and other compounds from the cell wall make difficult the extraction of these phenolic compounds. Thus, different strategies have been developed for the extraction and analysis of NEPs from plants.

4. ANALYTICAL METHODOLOGIES FOR THE EXTRACTION OF NEPs FROM PLANTS

4.1. Sample pretreatment before extraction

In order to guarantee the extraction efficiency of NEPs on the extraction process, a sample pretreatment prior to the extraction may be needed. There are different sample pretreatments according to the complexity of the sample matrix [33]. For instance, solid samples can be subjected to particle size reduction. It is important because the particle size affects mass transfer. Thus, for the purpose of increasing the contact surface of the matrix with the solvent for the recovery of NEPs, the most used technique is the milling using a centrifugal mill [34-37] or cutting mill [7, 38, 39], although in some investigations a grinder [40], crushing in a mortar [41] or press [42] were used (see Table 1). Subsequently, the samples are sieved to achieve a uniform particle size and to guarantee the extraction efficiency of NEPs. For instance, the optimum particle size used for the NEPs extraction from red and purple bran was 28 mesh screen [61], Cinnamoni Cortex between 60 and 80 mesh screen [61] and peanuts 60 mesh screen [63].

Also, the moisture content is important. Depending on the case, drying of the sample can induce contraction of the cells, which may decrease the extraction of the analyte from inside the cell. Therefore, swelling of the cell structure through moisturization of the raw material might be desirable [33]. . However, in some cases, the presence of water could decrease the stability of the sample because undesirable chemical and enzymatic reactions can happen due to water content. There are different procedures employed to reduce the water content of the sample to extract NEPs such as air-drying [34] which requires usually long times and high temperatures that can cause degradation of phenolic compounds. That is why freeze drying [38, 43, 45] and vacuum drying [48] are being used because they maintain the maximum amount of NEPs without degradation.

4.2. Extraction of NEPs from plants

Figure 3 shows the analytical process usually followed to carry out the extraction and characterization of NEPs. As it can be seen in Figure 3, once the sample has been pretreated, it is ready to undergo the extraction process. In order to give a clear overview on the techniques that are used to extract NEPs from plants, conventional and advanced extraction techniques have been described [48, 49, 60, 61]. However, the extraction might be incomplete due to strong interactions of NEPs with the matrix, as described in section 3. Therefore, sometimes the conventional and advanced extraction techniques produce an incomplete extraction by the impossibility of recovering NEPs and they are considered a sample pretreatment for carrying out the extraction of EPPs before the release of NEPs. Then, the extraction residue is treated with acid, alkaline or enzymatic hydrolysis in order to release NEPs [35, 34, 47]. Therefore, in this section the different extraction methodologies and residue treatments (acid, alkaline and enzymatic hydrolysis) employed for achieving the release of NEPs from the matrix are described.

4.2.1. Conventional extraction techniques

At present, conventional extraction techniques are the most commonly employed to carry out the extraction of NEPs from plants (see Table 1). They are based mainly on the use of organic solvents being solid-liquid extraction (SLE) the most widely used for solid samples [7, 40, 45] and liquid-liquid extraction (LLE) when the samples are liquids [54]. In both cases, the matrices are homogenized and extracted with a solvent such as methanol [40, 48] or a mixture of solvents such as methanol/water followed by acetone/water [35, 55].

Also, conventional extraction techniques were employed to extract EPPs previously to NEPs extraction from the matrix as a clean-up step. The extraction of EPPs from vegetable matrices was carried out with different types of solvents. The most used

solvents to extract EPPs have been acidic methanol/water (50:50, v/v; pH 2.0) followed by acetone/water (70:30, v/v) [44, 45, 47, 58]. In this sense, Tow et al. [64] showed that 50% ethanol was most effective to extract EPPs than using 80% methanol or 80% ethanol in combination with HCl (0.18N). However, the extraction yields of NEPs using butanol/HCl after the extraction of EPPs were lower when the EPPs extraction was carry out with 50% ethanol than with the methods which used 80% methanol or ethanol with HCl.[64]. The possible explanation is that the high efficiency of 50% ethanol could have extracted most of the polyphenols from the sample, being the best solvent for the extraction of EPPs. Aqueous-organic solvents with acidic pH are normally used because polyphenols are generally more stable at low pH, and it helps polyphenols to stay neutral without hydrolysing. So, the degradation of NEPs is smaller with low pH compared to high pH, since higher amounts of NEPs were obtained when using HCl [64]. Thus, the extraction of NEPs is done using a low concentration of a strong acid such as HCl (<1.0%) [63, 65]. However, these techniques are being replaced by new techniques that improve negative aspects of conventional extraction methods such as long extraction times, large amounts of organic solvents used or the low reproducibility and selectivity, among others [66].

4.2.2. Advanced extraction techniques

Conventional extraction techniques are being replaced by techniques which offer different advantages. New advanced extraction techniques have been developed which are faster, automated, and with improved reproducibility and selectivity compared to conventional extraction techniques. In addition, these techniques use more environmentally sustainable solvents [1, 66]. For instance, pressurized liquid extraction (PLE) [67] and ultrasound assisted extraction (UAE) [6, 46, 60, 67] have

been used as pre-process to clean the sample and to remove EPPs from the food matrix. Besides, pressurized hot water extraction (PHWE) [63], UAE [48, 49, 61], microwave-assisted simultaneous distillation and dual extraction (MDDE) [61], and supercritical fluid extraction (SFE) [68] have been employed to recover NEPs from plants.

As it can be seen in Table 1, the most used advanced extraction technique to recover NEPs has been UAE [48, 49]. Moreover, UAE was used to release NEPs through hydrolysis from cauliflower leaves, red cabbage and Brussels sprout waste streams [48].

Recovery of NEPs through UAE was influenced by several factors such as the type of solvent, the ratio of solvents, the pH or the extraction time. Different solvents and solvent mixtures were used to extract NEPs from plants, for instance, methanol/water (80:20 and 50:50, v/v) and acetone/water (70:30, v/v) [6, 49, 60]. In addition, the type and ratio of solvents have an influence on the extraction yield, and the degradation of phenolic compounds depends on the pH of the extracting solvents [47]. In fact, Cheng et al. [47] evaluated the influence of the solvent type, ratio of solvents and pH on the NEPs extraction with UAE from leaves of blueberries. The extraction solvent of 50% methanol in water followed by 70% acetone in water at low pH extracted higher contents of NEPAs than 70% ethanol in water at high pH [47]. Regarding the extraction time, this parameter varies depending on the matrix, the extraction method and the type of application [33]. Different works showed that the most common extraction times in UAE were between 15 min and 1 h for the extraction of NEPs from grape stems and blueberry leaves [6, 47, 49].

On the other hand, UAE was compared with PLE to extract EPPs as a pretreatment to extract NEPs from barley flour [67]. The PLE conditions were ethanol/water (4:1,

v/v) as extraction solvent, two extraction cycles of 5 min each, 60 °C and 20 MPa, whereas the extraction time was 10 min for UAE. Before the extraction, the residue was hydrolysed with 100 mL of 2 M NaOH at room temperature during 4 and 20 h, and with 6 mL of 96% ethanol and 30 mL of 25% HCl at 65°C for 30 min. Finally, the extracts obtained with PLE presented lower total phenolic content (TPC) and antioxidant capacity values than UAE. PLE had higher extraction yields but it was less selective than UAE because the diffusivity coefficient and the extraction power of a liquid increase in PLE [67].

Furthermore, the conditions for the extraction of PAs from Cinnamomi Cortex by MDDE were optimized [69]. Different solvents (ethyl ether, dichloromethane and petroleum ether) were compared, being ethyl ether the most effective solvent. The optimal extraction conditions were liquid-solid ratio of 18.0 mL/g, microwave irradiation time of 38 min and microwave irradiation power of 374 W at a temperature of 35°C. The MDDE method developed presented higher extraction yields of PAs in shorter times than conventional extraction techniques such as hydrodistillation [69].

SFE and PHWE were compared to Soxhlet for the recovery of hydrolysable tannins from *Phyllanthus niruri* Linn [68]. PHWE at 100°C of temperature and 10 MPa of pressure during 0.6-0.8 h produced higher extraction yields than Soxhlet using ethanol/water (30%, v/v) for 3 h. This may be due to the reduction of the water polarizability/polarity and the increase of solubility of corilagin (an ellagitannin) under PHWE conditions. Additionally, SFE with CO_2 as solvent and water and ethanol/water such as co-solvents was studied. Supercritical CO_2 with 10% (v/v) ethanol/water (50:50, v/v) as co-solvent at a flow rate of 1.5 mL/min, 100°C of temperature and 20 MPa of pressure were the best extraction conditions for the extraction of hydrolysable tannins. However, PHWE was recommended for the

extraction of hydrolysable tannins due to shorter extraction times, although this technique requires more quantities of solvent than SFE [68].

In general, NEPs remain bound to residue after the use of these extraction techniques. Thus, some researchers developed different treatments of the residue after the extraction of EPPs to recover the compounds of interest (NEPs) (see Figure 3) [17, 40, 45, 48].

4.2.3. Residue treatment

As described above, conventional and advanced extraction techniques produce an incomplete extraction by the impossibility of recovering NEPs due to strong interactions of these compounds with the matrix [64]. Thus, after extraction, a residue treatment is necessary for releasing NEPs trapped in the matrix using acid, alkaline or enzymatic hydrolysis [1, 17].

Moreover, changing the pH of the medium and using enzymes can help to break down non-covalent complex bonds between the analyte and the matrix in order to release NEPs [6, 51, 70]. However, conventional acid or alkaline hydrolysis, as well as enzymatic hydrolysis, should be used carefully given the risk of breaking covalent bonds in the polyphenols.

For instance, the acid hydrolysis causes break in glycosidic bonds and dissolves sugar moieties, although it generally leaves ester bonds intact [29, 70]. Nevertheless, acid hydrolysis at high temperatures can result in the loss of some phenolic compounds [30] and cause the depolymerization of HTs producing their degradation into gallic and ellagic acids [71].

Table 1 shows that acid hydrolysis was frequently applied using butanol/HCl (95:5 and 97.5:2.5, v/v) in the presence of 0.7 g of $FeCl_3$ to release PAs or condensed tannins [35, 44, 47]. However, some authors employed butanol/HCl without $FeCl_3$ or

methanol/H_2SO_4 [6, 7, 17, 72]. The use of $FeCl_3$ depends on the analytical methodology employed since it is necessary to carry out a specific spectrophotometric method (see section 5.1.1) [35, 45].

Cheng et al. [6] observed that the acid hydrolysis with methanol/H_2SO_4 produced higher extraction yields of NEPs from blueberries than ethanol/H_2SO_4. Furthermore, the extraction temperature and solid-liquid ratio had influence on the extraction yields of NEPs. In fact, high extraction temperatures and solid/liquid ratio provided lower extraction yields of NEPs, possibly due to the phenolic degradation. Thus, the optimal conditions in this study were solid/liquid ratio 1:20.7, extraction temperature 73.9°C and extraction time 22.7 h with methanol/H_2SO_4 (90:10, v/v) [6].

In addition, depending on the food matrix the optimal conditions for the extraction are different [44, 73]. In general, the most common conditions to carry out the acid hydrolysis of PAs have been 1 h for the treatment with butanol/HCl (95:5, v/v) at 100°C [4, 35, 44, 47].

On the other hand, alkaline hydrolysis breaks the ester bond linking phenolic acids on the cell wall releasing phenolic compounds from polysaccharides [30]. Therefore, this treatment does not produce the degradation of some phenolic compounds [30]. As it can be observed in Table 1, alkaline hydrolysis was frequently applied using different concentrations of sodium hydroxide between 2 and 4 M [17, 40, 48]. For instance, the release of NEPs from blueberries was higher when NaOH concentration increased, because the hydrolysis of polyphenols was accelerated [6]. NaOH was needed to liberate the NEP fraction because it can break ester-bonds, hydrolyse cell walls and solubilize proteins [40]. Furthermore, alkaline hydrolyses were usually carried out at temperatures from room temperature to 60°C and reaction times from 15 min to 4 h (see Table 1) [17, 40, 48].

In general, the residue treatment was carried out by stirring [35, 36, 45]. However, the effect of the combination of sonication and alkaline hydrolysis for achieving NEPs from cauliflower waste, Brussels sprout tops and stalks and red cabbage waste was studied [40, 48]. The combination of alkaline hydrolysis and sonication (frequency of 37 kHz and a nominal power of 180W) released higher amounts of NEPs from the plant matrix compared to sonication or alkaline hydrolysis alone [40, 48]. Additionally, the reaction temperature and time were important variables. For instance, an increase in temperature steadily yielded higher TPC. However, when the temperature was higher than 60°C, the TPC values were lower in Brussels sprouts stalks compared to temperatures less than or equal to 60°C, possibly due to that NEPs from Brussels sprouts stalks may be more thermally labile compared to the NEPs from other matrix such as Brussels sprouts top and red cabbage. With reference to time, the study showed no significant differences between 30 and 45 min of hydrolysis time for Brussels sprouts [40]. In this sense, the optimal conditions were 80°C, 4 M NaOH and 30 min for Brussels sprouts (top), 60°C, 4 M NaOH and 30 min for Brussels sprouts (stalks), and 80°C, 4 M NaOH and 45 min for red cabbage waste [40]. On the other hand, for the extraction of cauliflower waste, Gonzales et al. [48] observed that the optimal conditions were 2 M NaOH at 60°C for 30 min of sonication.

In general, acid hydrolysis can contribute to higher extraction yields than alkaline hydrolysis. Additionally, acid hydrolysis may be useful to obtain an amount of polyphenols closer to the real ones, although it is a more aggressive treatment since it causes the degradation of some compounds [17, 67]. Both hydrolysis, acid and alkaline, are non-specific and might change the conformation of NEPs being very difficult to know their real structure. Therefore, a more specific, non-toxic and

effective method to release NEPs is needed such as enzymatic hydrolysis, although it is not very commonly used for the extraction of NEPs. Thus, the enzymes glucuronidases, pectinases, cellulases, hemicellulases, glucanases, tannases and amylases have already been employed to break the carbohydrate linkages and to decompose the cell wall structure [51, 74]. However, the use of enzymes in different applications have been limited, due to the fact that enzymes need usually mild conditions of pH, temperature and pressure, because they are unstable under drastic conditions of these parameters [72]. Commercial enzyme preparations were employed to release polyphenols from grape skins and seeds such as pectinase, cellulose and tannase [50]. Some authors have shown that depending on the enzyme used, different NEPs can be extracted [5, 74]. For example, Pérez-Jiménez et al. [5] reported that a combination of cellulose and peptinase exhibited higher yields of NEPAs than esterase in apple.

A number of factors could have influence on the enzymatic hydrolysis yields. Thus, the proportion of cellulose and lignin present in the plant matrix had a considerable influence since the combination of cellulose and lignin produces a material highly resistant to enzymatic degradation. In fact, higher content of cellulose and lignin and the presence of fiber in the matrix might decrease the release of NEPs [50]. Therefore, from the comparison of enzymatic and acid hydrolysis, it was observed that the enzymatic hydrolysis recovered lower amounts of NEPAs from cereal samples than acid hydrolysis [5]. However, enzymatic hydrolysis may promote the discriminated release of NEPAs, being this type of hydrolysis more selective than acid hydrolysis [50]. Nevertheless, the combination of both treatments, acid and enzymatic hydrolysis produced a greater polyphenol release than their separate use [5].

The residue treatment enables the recovery of NEPs that remain retained on the matrix increasing the extraction yields of phenolic compounds and avoiding the underestimation of these compounds in later analysis (see Figure 3) [17, 45].

4.3. Purification

A wide range and combination of techniques have been used in order to isolate NEPs (see Figure 3). For instance, solid-phase extraction (SPE) on columns of hydroxypropylated dextran gel (i.e. sephadex LH-20) [36, 61], vynil polymers such as Diaion HP-20 or silica (C-18) [40, 48, 75] (preconditioned with water or methanol) allowed the removal of sugars and other highly polar compounds (such as organic acids, amino acids, proteins, among others). These compounds were eluted with water, methanol, ethanol and their combinations for C-18 and Sephadex LH-20 cartridges [36, 40, 53, 61], eventually with 0.1% formic acid for C-18 cartridge [40, 75]. Then, phenolic compounds can be recovered by elution with methanol [40] or aqueous acetonitrile with C-18 cartridges (1:1, v/v) [73], and aqueous acetone with LH-20 cartridges [36, 61]. Finally, samples are dried for subsequent analysis [61]. Also, LLE can be used to enrich high molecular weight NEPs and to wash extracts using butanol or water [7, 41, 47] or their combinations [55].

SPE and LLE cannot separate the different types of phenolic compounds, for instance polymeric polyphenols from other phenolic compounds. That is the reason why other methods have been used with the aim to isolate polymeric polyphenols from other phenolics, as well as to fractionate them depending on the DP of the compounds of interest [34]. In this sense, preparative-HPLC with a YMC-Pack ODS-A column was used to achieve the separation of monomeric and trimeric PAs from grape seeds. Isocratic elution was employed with two solvents, water (solvent A, 100%) and methanol (solvent B, 100%) and different combinations [34]. Also, the isolation of

monomeric, dimeric and trimeric PAs from grape seeds was carried out by preparative high-speed counter-current chromatography separation (HSCCC) with a two phase solvent system composed by n-hexane-ethyl acetate-water (1:50:50, v/v/v) [34]. The two phases were separated and the upper phase was used as the mobile phase and the lower phase as the stationary phase. The HSCCC was rotated at 950 rpm for 1 min, and the upper mobile phase was pumped into the column at a flow rate of 3 mL/min and 20 mL of the lower phase with 400 mg of sample with PAs was injected [34]. The PAs were separated according to the DP (see Figure 4).

These methodologies represent an attractive alternative for the isolation of polymeric polyphenols according to the DP.

5. QUANTIFICATION AND CHARACTERIZATION OF NEPs

There are not many commercially available oligomeric and polymeric polyphenol standards, and usually they are very expensive. This is one of the main reasons that make difficult the preparative and analytical chromatographic separation, quantification, and structural characterization of oligomeric and polymeric polyphenols. For instance, it seems problematic for a valid quantification that in many cases calibration standards with the monomer were used, which do not necessarily reflect the polymeric polyphenols analyzed in the test samples. Additionally, analytical quantification of tannins is typically achieved by colorimetric methods. These methods are not even comparable with the absolute polymeric polyphenols content because of the use of reference standards for calibration which are not related to the polymeric structures; also, in many cases these methods are not selective for polymeric polyphenols, and other compounds can react with the reagent used giving a false positive.

In this section, the different methodologies employed to carry out the quantification and identification of NEPs are discussed.

5.1. Quantitative analysis

5.1.1. UV spectrophotometric methods

At this moment, there are several spectrophotometric methods to determine total polyphenol content. The most commonly employed method for this measure is Folin-Ciocalteu assay [74]. It is well known that, this assay may overestimate the content of phenolics in the sample in cases in which there are other compounds with reducing groups that can also transfer electrons to molybdenum, being nonspecific to phenolics [76, 77].

The determination of the content of PAs in the extracts has been carried out by different assays such as dimethylaminocinnamaldehyde (DMAC) [43, 45], vanillin [6] and proanthocyanidin assays [44, 45], among others.

Concerning DMAC assay, the reaction mechanism is not clearly defined. However, it seems that the DMAC reagent might react with the flavonoid molecules which have free meta-oriented hydroxyl groups and with a single bond at the 2,3-positions of the C-ring [78]. Also, it has been suggested that DMAC reacts with one monomeric unit in each proanthocyanidin molecule, although this has not been clearly demonstrated. Nevertheless, large polymeric compounds may not be detected with as much sensitivity as monomeric compounds with the DMAC reagent [78].

Vanillin assay consists in the reaction of vanillin with the flavonoid ring at the 6 or 8 positions. This method is less specific than the DMAC assay because acid conditions and concentration of substrate in the reaction medium may influence the analysis [76].

In addition, proanthocyanidin assay is the most employed for measuring total content of PAs. This assay is carried out in a solution of butanol-concentrated HCl with $FeCl_3$

[6, 55]. Under these conditions the PAs are converted into anthocyanidins by the presence of HCl, and these compounds are measured to 555 nm. Some authors performed the absorbance measurement at 555 and 450 nm in order to detect anthocyanidins and xanthylium compounds, respectively [35, 44, 45]. The use of $FeCl_3$ is due to the fact that Fe^{2+} and Fe^{3+} are the most effective catalysts for the conversion of PAs to anthocyanidins [77].

On the other hand, KIO_3 assay was used to carry out the quantification of the total hydrolysable tannins [59]. This assay consists of the reaction between KIO_3 and hydrolysable tannins. Hydrolysable tannins react with KIO_3 to produce a purple colored product. Subsequently, the reaction product is measured at a wavelength of 525 nm [59]. However, the KIO_3 assay presents some limitations due to variable reaction times necessaries for achieving the maximum color yield. Also, the formation of yellow oxidation products from other phenolic compounds could interfere with the measurement [79].

The rhodanine assay has been employed for the estimation of gallotannins and sodium nitrite assay for the quantitative determination of the ellagic acid [22, 80].

KIO_3, rhodanine and sodium nitrite assays are scarcely used, possibly due to the interferences from other compounds in the measurement. Thus, the information about the identification of NEPs through these assays is very limited [79].

Spectrophotometric assays have several benefits such as being easy to perform and the low cost of the experiments [1]. However, these methods only give an estimation of the total phenolic compounds and they do not provide a quantitative measurement of individual compounds [1]. Consequently, in the last few years, some authors focused their efforts on the use of chromatographic techniques for qualitative and quantitative analysis of NEPs [17, 39, 60, 81].

5.2. Qualitative analysis

5.2.1. Chromatographic techniques

The analytical separation of polymeric polyphenols by HPLC has been described in the literature [42, 45, 81]. The chromatographic separation depends on the respective molecular weight, stereochemistry, polarity and the secondary modification of the analytes which implies that different individual methods have to be applied. In the literature, mainly two different kinds of stationary phases for the separation of NEPs were reported, reverse phases (RP) and hydrophilic normal phases (NP) (see Table 1). NP-HPLC is usually selected for having a good resolution for PAs up to a DP 10-12. For instance, NP-HPLC was chosen for the separation of the procyanidins oligomers from chocolate and cocoa [82]. Also, White et al. [36] used NP for the separation of monomers, dimers and trimers procyanidins from cranberry pomace using a silica column. Unfortunately, NP-HPLC does not pair with ESI, and chemical ionization methods are required [75] which originates lengthy preparation times to obtain structural information [82]. That is why, NP-HPLC is usually coupled to fluorescence detector (FLD) because it has the best sensitivity for the detection ofPAs. However, relative fluorescence response factors decrease with increasing DP [80].

Thus, usually, RP-HPLC has been employed to characterize NEPs [4, 11, 17, 40, 68, 83]. Phenolic compounds are eluted according to their polarity [75]. Nevertheless, the determination of PAs by RP-HPLC may be problematic in complex samples because their separation is difficult and it could produce peak overlapping [46]. In order to improve the identification by RP-HPLC of PAs, sometimes thiolysis or phloroglucinolysis methods are carry out. These methods consist of breaking interflavane bonds in acid medium with the presence of a nucleophilic reagent, such as benzyl mercaptan or phloroglucinol, respectively [81]. The nucleophilic reagents

convert flavan-3-ol extender units into the corresponding benzyl thioether or phloroglucinol adducts, whereas the terminal units are releasing as underivatized monomeric flavan-3-ols. The stoichiometry of this reaction facilitates the determination of the mean DP from molar concentrations of derivatized and underivatized monomers as measured by HPLC [46]. For instance, the thiolysis method was employed to carry out the characterization of PAs from persimmon pulp, pinus, apples, berries, red and green grapes, *Averrhoa bilimbi*, and *Spatholobus suberectus* [39, 60, 68, 83, 84]. On the other hand, analysis by RP-HPLC of PAs from grape seeds and skins was achieved by phloroglucinolysis method [34, 74]. The use of phloroglucinol as nucleophilic reagent was more attractive than benzyl mercaptan, because phloroglucinol is odorless and more selective in the formation of 3,4-trans adducts from 2,3-trans flavan-3-ol extension units, desired for the HPLC analysis [85]. However, benzyl mercaptan provides unpleasant odor and its use is limited because it needs special handling conditions [85]. Besides, benzyl mercaptan results in lower yields of polymerization product than phloroglucinol [86]. In general, the main limitation of thiolysis is that some PAs resist to the breakdown of interflavane bonds, especially when PAs are from aged tissues such as outer bark. This limitation causes an elevation of the baseline due to the presence of polymeric PAs in the analysis by HPLC [86].

In RP-HPLC analysis, in general, a linear gradient elution with a binary system with different solvents has been employed for the separation of NEPs [45, 61, 74]. The mobile phases have been different depending on the phenolic type and the matrix. For instance, the most common mobile phases were acidified water and methanol or acetonitrile, and the elution gradient starts with high content of acidified water increasing more nonpolar acidified solvents such as methanol or acetonitrile. The

most commonly employed acids for the acidification of the mobile phases to pH around 2-4 have been formic or acetic acids [64, 83]. Also, other acids have been employed, such as trifluoroacetic acid for the separation of PAs and hydrolysable tannins from chestnuts peel [56]. However, the acidification of the mobile phases was not always needed, for example, to carry out the separation of procyanidins from *Averrhoa bilimbi* fruits and leaves [39].

HPLC instruments are very often coupled to UV/Vis or diode array (DAD) detectors, in which phenolic compounds in general are detected at 240-285 nm, hydroxycinnamic acids at 320 nm, flavones and flavonols at 350-365 nm and anthocyanins at 460-560 nm [17, 45, 87]. However, the DAD detection of PAs has some disadvantages. The spectra of monomers, procyanidin oligomers and their degradation products do not provide any differential information. In fact, diastereomers such as epicatechin and catechin cannot be distinguished exactly [75]. In addition, this type of detection does not provide information about the structure of individual compounds [83].

In order to carry out a structural characterization of NEPs, HPLC is coupled to a mass spectrometry (MS) detector (see section 5.2.2) [17, 39, 81].

Pérez-Jiménez et al. [45] developed an interesting separation method by RP-HPLC with a Luna C18 column to characterize with DAD hydrolysable tannins from different fruits. The hydroxibenzoic and hydroxicinnamic acids were the main constituents of hydrolysable polyphenols fraction in all fruits analyzed. Additionally, flavonols were detected in hydrolysable tannins fraction in apple, broccoli, cucumber, onion, pepper and red beetroot; and flavanones in mandarin [45]. On the other hand, Fernández et al. [50] carried out with a phloroglucinolysis followed by a separation of PAs from grape skin and seeds by RP-HPLC with UV detection. Then, the resulting

underivatized monomeric flavan-3-ols and terminal units were analysed using two columns C18 connected in series. It was possible to determine the phenolic composition depending on the DP, galloylation percentage and subunit. In fact, subunits of epigallocatechin-phloroglucinol, epicatechin-phloroglucinol, epicatechin gallate-phloroglucinol and catechin-phloroglucinol were separated [50]. Furthermore, Arranz et al. [17] observed by RP-HPLC-DAD that one of the main constituents of hydrolysable polyphenols fraction from wheat flour and a mixture of cereals was caffeic acid. Also, these authors identified other compounds such as epicatechin or gallic acid and their derivatives. Therefore, RP-HPLC methods provide important information about the individual compounds present in a sample or the structural changes that they could suffer by the residue treatment [17, 61, 74]. Unfortunately, as mentioned above, RP-HPLC is limited for the separation of larger PAs, and it can only separate individual PAs from monomers up to trimers [86]. Moreover, the peak capacity of the stationary phase is reduced in complex samples since it produces unresolved hump or a drift of the baseline can be observed [75].

However, new developments appeared in chromatography such as ultra-high performance liquid chromatography (UHPLC) and multidimensional liquid chromatography (LCxLC) [34, 40, 43]. In fact, UHPLC was used to separate NEPs from cauliflower waste, red cabbage and Brussels sprout waste streams [40, 48]. The separation was made with a RP column, and the mobile phase consisted of water with 0.1% formic acid and methanol with 0.1% formic acid. This separation allowed the identification of different phenolic compounds of the NEP fraction. This fraction was composed dominantly in the three vegetal matrices by flavonoid glycosides being kaempferol-3-*O*-diglucoside-7-*O*-glucoside the most abundant [40, 48]. Also, quercetin-7-*O*-glucoside and quercetin-7-*O*-diglucoside were found abundant in the

NEP fraction in red cabbage. However, quercetin-7-*O*-diglucoside was found in the NEP fraction but not in the extractable fraction from Brussels sprout [40]. Whereas, sinapic acid was the most abundant phenolic acid in cauliflower waste [48].

Also, PAs from grape seeds were separated by UHPLC with a RP column and the mobile phase consisted of 0.2 % formic acid in acetonitrile and 0.2 % formic acid in water [34]. The analytical method enabled the separation of galloylated PAs dimers such as procyanidin B2-3-*O*-gallate, procyanidin B1-3-*O*-gallate, procyanidin B2-3´-*O*-gallate and proanthocyanidin trimer epicatechin- epicatechin-catechin). Also, non-galloylated proanthocyanidin dimers such as procyanidins B1, B2, B3, B4 and B8 were separated.

On the other hand, Montero et al. [43, 49] used two dimensional liquid chromatography (LC x LC) combining a HILIC column in the first dimension with a RP-C18 column in the second dimension (see Figure 5.a and 5.b). In the first dimension with the HILIC column, the procyanidins polymers on grape seeds and apples were separated depending on their DP and of galloylation (see Figure 5.a), while in the second dimension with a RP column the separation was according to the polarity of the phenolics. The mobile phases employed were acetonitrile with acetic acid (98:2, v/v) and methanol with water and acetic acid (95:3:2, v/v/v). The LC x LC was hyphenated to a DAD and an Ion Trap MS detector operating under negative ESI mode, which allowed the tentative identification of the phenolic compounds with a mass range, m/z 90-2200 Da. The developed methods enabled a separation in less than 50 min and a tentative identification of 65 compounds in the different types of apples, including flavan-3-ols oligomers up to a DP 8, dihydrochalcones, flavanols and phenolic acids, and 46 compounds in grape seeds including flavan-3-ol oligomers up to a DP 7 [43, 49]. The increase in peak capacity brought by the two-dimensional

system enabled the analysis of plant-based samples of large complexity. Therefore, the multidimensional liquid chromatography online coupling together with DAD and MS has shown to be a promising technique to carry out the separation and identification of NEPs with a limitation of the mass to 2200 Da [43, 49].

On the other hand, HPLC is preferred over gas chromatography (GC), because in the latter phenolic compounds need to be derivatized to volatile compounds by methylation, trifluoroacetylation or conversion to trimethylsilyl derivatives before being analyzed because they have few volatility [1, 61, 88]. That is why, there is scarce information on the analysis of NEPs by GC. In the last few years, GC has only been used to determine oligomeric and polymeric PAs from Cinnamomi cortex. A MDDE extraction to obtain and facilitate the extraction of volatile compounds was performed. Subsequently, GC enabled the separation of oligomeric and polymeric PAs from Cinnamomi cortex without a previous derivatization [61].

5.2.2. Mass spectrometry

MS has been used directly, without a previous chromatographic separation, to carry out a qualitative analysis of NEPs [53, 60], although this technique is commonly used coupled to HPLC (see section 5.2.1) [39, 81].

Different ionization sources are usually employed for MS analysis of phenolic compounds such as, fast atom bombardment (FAB), electrospray ionization (ESI), atmospheric pressure photoionization (APPI) and matrix-assisted laser desorption ionization (MALDI) [1]. In this sense, in the last few years, researchers have been employed ESI [41, 42, 45] and MALDI for the analysis of NEPs [36, 38, 60].

ESI-MS can be applied to detect thermosensible analytes with low and medium molecular weight. This technique makes it possible to discriminate among classes of polyphenol families and gather information on the glycosylation position. In general,

sensitivity is greater with negative ionization where multiple deprotonated ions are formed and procyanidins olygomers with molecular weights greater than tetramers detected the multiple charged ions [62, 69].

HPLC-DAD has been coupled to ESI-MS (operating in ion negative mode) for the characterization of NEPAs from plant materials [4, 68] and cereals [17]. This technique provides information about the qualification and quantification of individual oligomers such as (epi)catechin gallate benzylthioether [46], *p*-hydroxybenzoic acid [4] and caffeoylquinic acid [17], among others, from the different matrices studied.

However, the poor ionization of larger NEPs using ESI is a limiting factor for the identification of NEPs. Usually bias toward the smaller NEPs are introduced because poor or no detection of larger molecules. It is problematic in qualitative and quantitative analysis by MS because of the lack of authentic standards [54, 77]. In addition, the interpretation of spectra with ESI is difficult because it provides multiply charged ions from polydisperse PAs and it does not have the capacity of interpret each of them. Furthermore, some MS instruments are limited by their upper mass range (typically around 4000 Da) [87]. In contrast, MALDI offers several benefits compared to ESI since MALDI provides a greater tolerance for impurities, detects single charged ions and permits reanalyze the same sample [87]. Besides, MALDI has the possibility to be coupled to analyzers with unlimited mass range [87].

There are different mass analyzers which are sector analyzers (electric E and/or magnetic B), quadrupole (Q), time of flight (TOF) and ion traps (Ion Traps, Orbitraps and ion cyclotron resonance ICR) [88]. Generally, MALDI is usually coupled to time-of-flight (TOF) MS. MALDI-TOF/MS has been a common employed technique for the analysis of polymeric NEPs because it does not have theoretically limited mass

range [1, 36, 38, 89]. The analyzer TOF separates and detects on a time-scale (flight time) full package of ions from the source. The system works for this reason in discontinuous regime so it is a suitable detector for pulsating ionization like MALDI. Nevertheless, other systems as Q or sector analyzers filter groups of ions in each instant with a narrow range of values m/z discarding the rest of ions, thus these systems are less appropriate than TOF [88]. For instance, MALDI-TOF/MS has been successfully applied for the analysis of NEPAs from monomers up to heptamers [1, 36]. Moreover, MALDI-TOF/MS allows detection of high mass with accuracy and high resolution, being more suitable than ESI for the analysis of high molecular weight compounds such as NEPs, providing better detection in positive ionization [52]. In fact, PAs profile from *Uncaria tomentosa* L. (cat´s claw) was determined by ESI-QTOF/MSwhich enabled the identification of procyanidins with a DP between 1 and 10 units. However, compounds with a DP greater than 10 cannot be detected [62] while MALDI-TOF/MS can detect compounds with a DP greater than 10 [90]. These studies confirm that MALDI-TOF/MS is a potent tool for qualitative analysis of procyanidins and provides the identification of phenolic compounds with a high DP since it has unlimited mass range of detection by the instrument [62, 77, 89, 91]. The combination of ESI-QQQ/MS and MALDI-TOF/MS analyses was excellent for the identification of ten procyanidin fractions with different DP (from trimers to 11-mers) from *Spatholobus suberectus* [60]. ESI-QQQ/MS enabled the identification of procyanidin monomers and their oligomers, while MALDI-TOF-MS besides allowed the analysis of procyanidins from tetramers to 11-mers [60]. As an example, Figure 6 shows a MALDI-TOF-MS spectrum of the procyanidin fractions from *Spatholobus suberectus*. This spectrum clearly demonstrates the kind of information that can be obtained [60].

As mentioned above, the most common mass analyzer used for the determination of NEPs has been TOF [36, 37, 53, 60, 92]. However, Montero et al. [43, 49] have employed Ion Trap mass spectrometer equipped with an electrospray interface to identify different monomers and procyanidin oligomers from grape seeds and apples. Also, Fischer et al. [42] employed Ion Trap mass spectrometer with an ESI source to determine HTs from pomegranate. Ion trap has various advantages since it permits the tenure of ions inside a chamber with electric fields during prolonged times facilitating the observation of breakdowns of the compounds. This trap allows the isolation of ions individually. In addition, Ion Traps can use different ionization sources such as MALDI, ESI or FAB [88].

MS is an analytical technique which represents an attractive alternative for elucidating the chemical structures of molecules such as NEPs [1]. However, other techniques are nowadays being used with this aim to analyse NEPs such as nuclear magnetic resonance (NMR) [1, 77].

5.2.3. Nuclear Magnetic Resonance

The structural characterization of NEPs can also be achieved by NMR. The advantages of NMR spectroscopy are recognized for simplifying sample pretreatment and measurement procedures, its high resolution, being nondestructive, or conferring the possibility to analyze liquid or semi solid food. All this makes NMR a powerful and attractive technique for structural characterization of NEPs compared to MS [1]. However, the information about the analysis of NEPs through NMR is very scarce, possibly due to the cost of equipment and limited studies of these compounds. The studies related to the analysis of polymeric polyphenols from vegetables show that NMR could be a versatile methodology since it provides different advantages that other techniques may not provide [1, 34].

Various NMR techniques have been used for the elucidation of structural complex phenolics such as ^{1}H and ^{13}C NMR, two-dimensional homonuclear (^{2}D ^{1}H–^{1}H) correlated NMR spectroscopy (COSY), totally correlated NMR spectroscopy (TOCSY), nuclear overhauser effect spectroscopy (NOESY), rotating frame of reference (ROESY), heteronuclear chemical shift correlation NMR (C–H HECTOR), heteronuclear multiple bond correlation (HBMC) and heteronuclear multiple quantum coherence (HMQC) [1, 57].

For instance, one dimensional NMR (1D) (^{1}H) was used to make a structural identification of PAs isolated by HSCCC from grape seeds and skin [34]. The structure of different monomeric, dimeric and trimeric PAs obtained after phloroglucinolysis treatment such as catechin, epicatechin, epicatechin-3-*O*-gallate, epicatechin-phloroglucinol derivative, catechin-phloroglucinol derivative, epicatechin-3-*O*-galloyl-phloroglucinol derivative, proanthocyanidin B2-3´-*O*-gallate, B3, B4, T2 and C1 was determined from grape seeds [34]. Also, the structure elucidation of 15 compounds from grape skin was possible (catechin, epicatechin, astilbin, gallic acid, procyanidin B2-3-*O*-gallate, procyanidin B1-3-*O*-gallate, procyanidin B2-3´-*O*-gallate, trans-coutaric acid, quercentin-3-*O*-glucuronide, procyanidin B1, B2, B3, B4, T2 and C1) [91].

On the other hand, two-dimensional NMR (^{1}H-^{1}H-COSY, HMQC, HBMC) was employed for the characterization of procyanidins from grape seeds [89]. Two-dimensional NMR provided more information about the molecular structure than one dimensional NMR. In this sense, the structural characterization of procyanidins B1, B2, B3, B4, B5 and B7 was performed. And it was possible to find differences in the chemical shifts of a number of protons in the spectra of some compounds, such as procyanidins B2 and B5 or procyanidins B1 and B7. The compounds were identified

using these types of differences [89]. Moreover, the operating temperature had influence on the NMR spectrum definition. In fact, high temperatures produced poorly defined spectra. Thus, in this study low temperatures of operation were used (30°C) [89].

In addition, NMR spectroscopy was used to confirm the mass spectrometric data and detect little differences in the structure of polymers from cinnamon [92]. The identification of little differences among compounds was essential to elucidate similar compounds [92].

These studies reflect that the use of NMR provides a rapid elucidation of polymeric polyphenols from plants. The structural characterization with these types of techniques is essential to get to know the structures of NEPs and to be able to correlate amounts present in plants with biological activities that some researchers have shown.

6. CONCLUSIONS

In order to release polymeric polyphenols or NEPs from the residue of extraction different hydrolysis treatments (acid, alkaline or enzymatic) have been developed. In this way, hydrolysis allows the subsequent analysis of the compounds of interest. Regarding analytical methodologies, there are several spectrophotometric assays for the analysis of NEPs such as proanthocyanidin or vanillin assays. However, these spectrophotometric assays do not provide a measurement of individual phenolic compounds. Thus, the interest on the chromatography and mass spectrometry analysis of NEPs has arisen to identify individual compounds. In this sense, different mechanisms have emerged to enhance the identification of these compounds as HPLC-DAD-MS, HPLC-ESI-MS or MALDI-TOF/MS, among others. In fact, two-dimensional nuclear magnetic resonance has been postulated as a potent analytical

tool since it permits to obtain relevant information about the molecular structure of compounds.

In addition, these studies facilitate a complete database of methodologies for carrying out the analysis of NEPs from plants. However, the extraction of these compounds from their matrices as well as their qualitative and quantitative analysis are considered a challenge because the developed analytical methodologies present some limitations. In some cases, they are not sensitive enough, selective and specific and there are not many commercially available standards of oligomeric and polymeric polyphenols.

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Figure captions

Figure 1. Chemical structure of PAs (where catechin (R1 = H, R2 = OH, and R3 = H) and epicatechin (R1 = OH, R2 = H, and R3 = H)).

Figure 2. Chemical structure of a) gallic acid, b) gallotannin (1,2,6- trigalloyl glucose), c) ellagic acid and d) ellagitannin (pedunculagin).

Figure 3. Usual procedure to determine NEPs in plants.

Figure 4. Chromatogram corresponding to the HSCCC separation of PAs from grape seed according to the DP: F1, monomeric PAs; F2-6, oligomeric PAs; F7, oligomeric and polymeric PAs [34]. (Reprinted with permission from [34]).

Figure 5. a) HILIC chromatogram (280 nm) corresponding to the separation of phenolic compounds from apple in the first dimension of LC x LC; and b) two-dimensional plots (280 nm) corresponding to the separation using an optimized HILIC x RP method, modified from [43]. (Reprinted with permission from [43]).

Figure 6. MALDI-TOF/MS spectrum of the procyanidin fraction from *Spatholobus suberrectus*, modified from [60]. (Reprinted with permission from [60]).

Figures

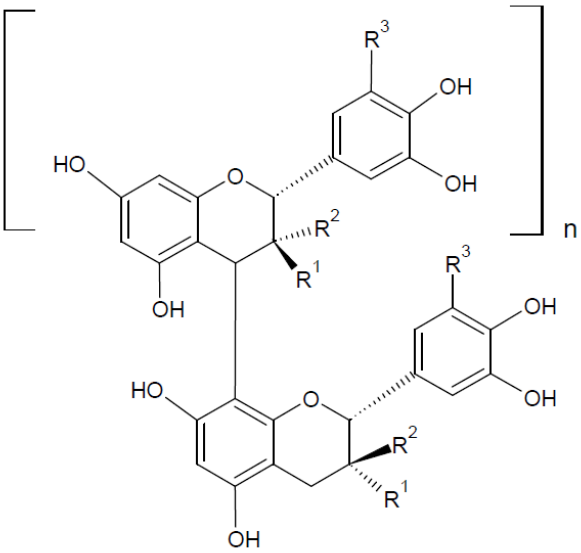


Figure 1.

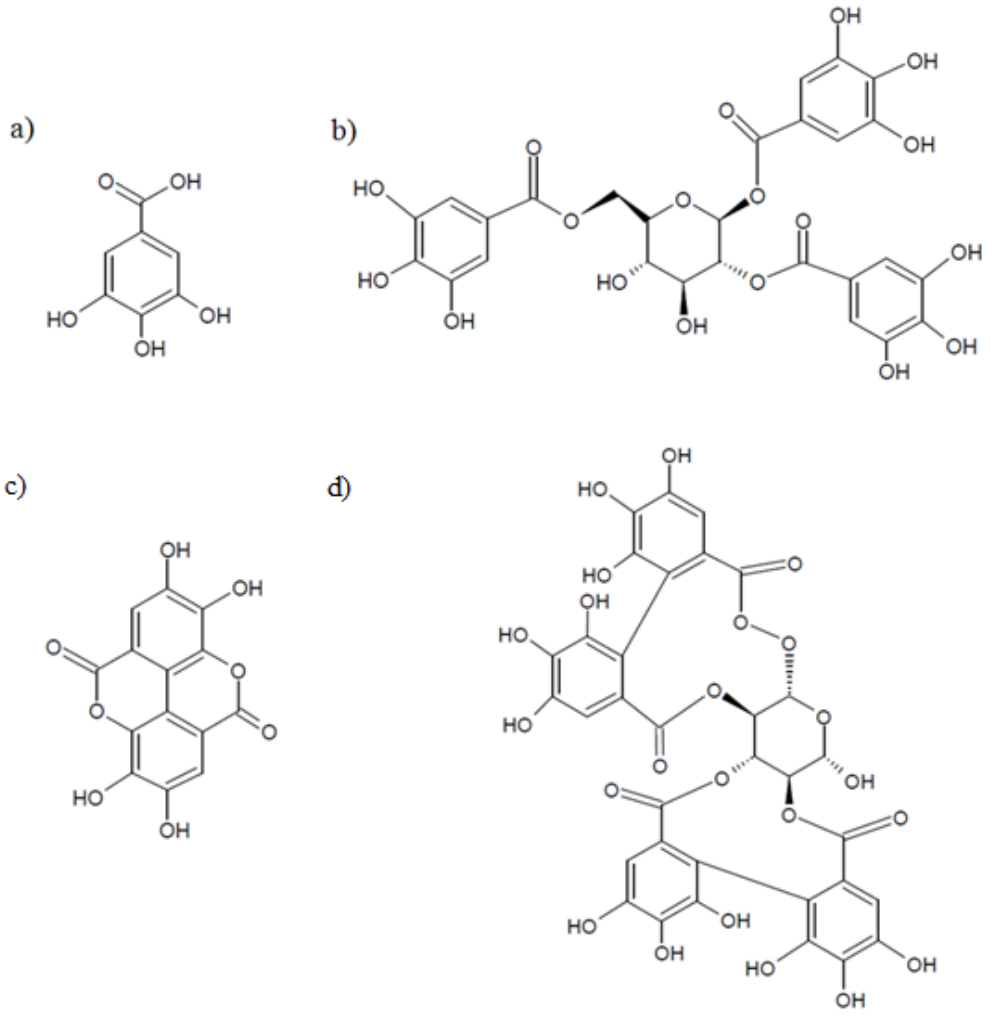


Figure 2.

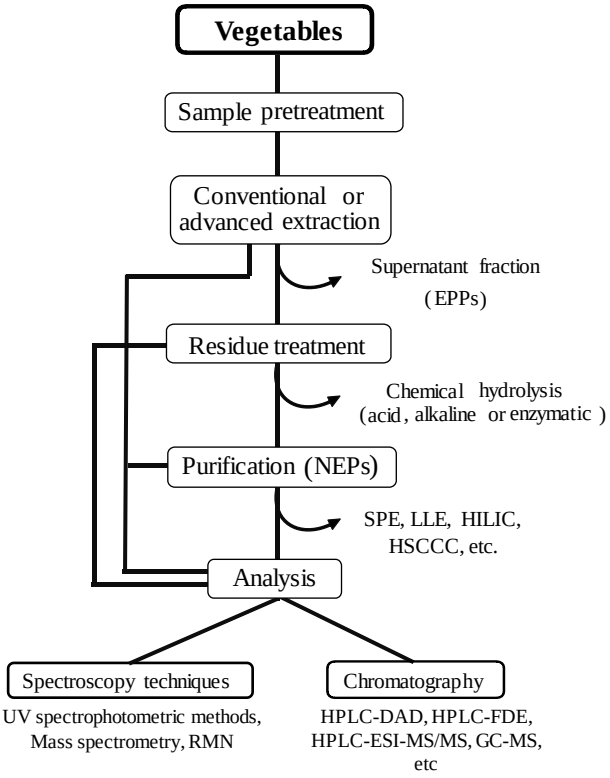


Figure 3.

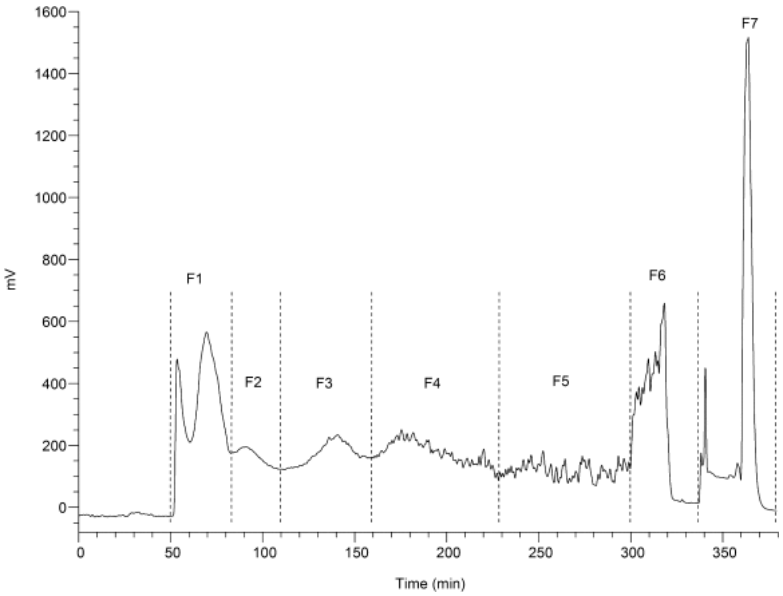
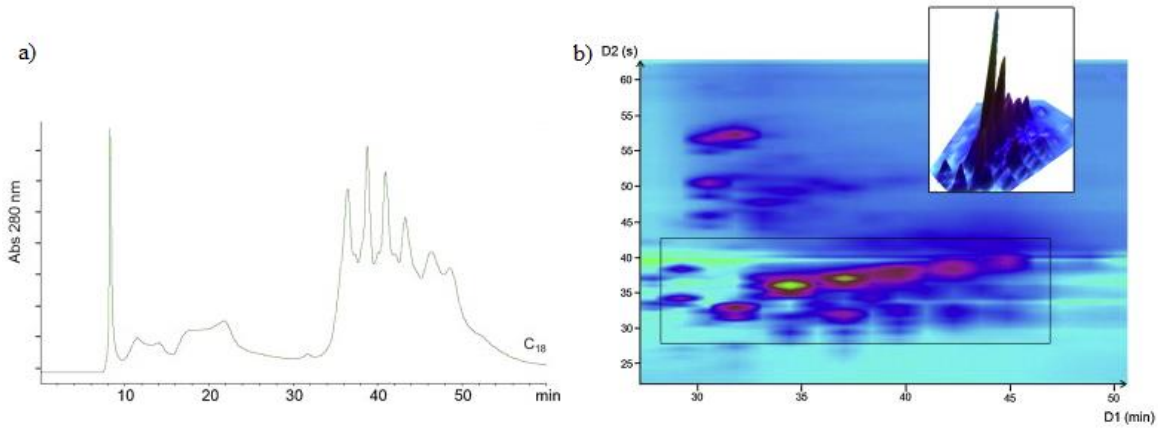


Figure 4.



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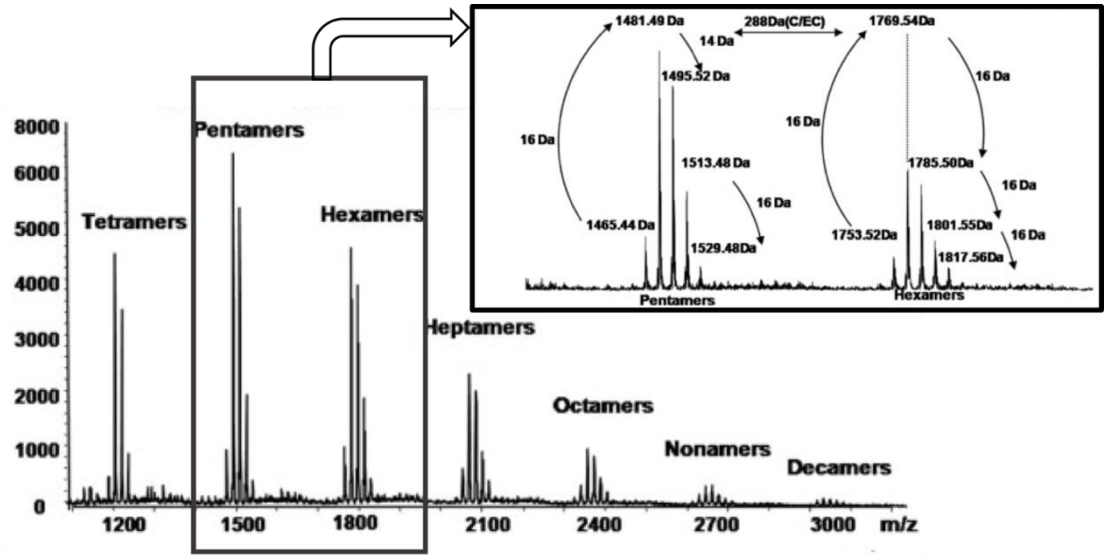

Figure 6.

Tables

Table 1. Non-extractable polyphenols analysis in food

Source and reference	Sample pretreatment	Sample preparation for analysis	Analytical method
Apple varieties [43]	Cutting and freeze-drying	Stirring for 20 min with acetone/H_2O (70:30). Repeated 2x with centrifugation in between. Evaporation and clean-up on SPE (DSC-18) column	Folin-Ciocalteu (TPC), DMAC (PAs), HILIC x RP-HPLC-DAD-ESI/MS (PP)
Apple (flesh and peel) [44]	Grinding. Homogenization (acidic methanol (HCl)/H_2O, 50:50, 1h, room temperature). Centrifugation. Extraction with acetone/H_2O by agitation (70:30, 1h)	Residue was hydrolysed with HCl/butanol (5:95) with $FeCl_3$ (90°C, 1h). Centrifugation	Folin-Ciocalteu (TPC), 555 nm and 450 nm (NEPAs).
Apple, peach, nectarine [4]	The edible parts were freeze-dried and milled to a particle size of 0.5 mm in a centrifugal mill. Extraction with methanol/water (50:50, v/v; pH 2) acidified with 2N HCl (1h, room temperature); Centrifugation; Extraction with acetone/water (70:30, v/v)	Residue was hydrolysed with HCl/butanol (2.5: 97.5 v/v) and 0.7 g of $FeCl_3$ (100°C, 60 min); Centrifugation	Proanthocyanidin assay 555 nm (NEPAs). HPLC-ESI-MS (NEPs)
Apple raw and apple peel (*Golden delicious*) Banana (*Cavendish*) Cooked pinto beans. Red grape pomace (*Cencibel*) [35]	Freeze-drying and milling. Extraction with acidic methanol/H_2O (50:50, 1h, room temperature) followed by acetone/H_2O extraction (70:30, 1h, room temperature), centrifugation and drying	Residue was hydrolysed with HCl/butanol (5:95) with $FeCl_3$ (100°C, 1h). Centrifugation.	555 nm and 450 nm (NEPAs).
Apple, asparagus, banana, broccoli, brussels sprouts, carrot, chad, chicory, courgetter, cucumber, grape, green bean, lettuce, mandarin, melon, onion, orange, peach, pear, pepper, red beetroot, tomato, watermelon, white cabbage [45]	Freeze-drying and milling. Homogenization with acidic methanol (HCl)/H_2O (50:50, 1h, room temperature). Centrifugation. Extraction with acetone/H_2O (70:30, 1h, room temperature) and centrifugation	Residue was hydrolysed with methanol/H_2SO_4 (pH 2.0, 20h, 85°C). Clean-up on SPE (Oasis HLB) cartridges. Concentration. Residue was hydrolysed with butanol/HCl with $FeCl_3$ (100°C, 1h)	Folin-ciocalteu (EPP, HPP), HPLC-DAD (C18 column) (HPP), 555 and 450 nm (NEPAs), HPLC-ESI-QTOF (C18 column) (HPP)
Apple, peach, pear, nectarine, white grape, strawberry, banana, cranberry beans, cocoa powder, baby apple, baby banana [5]	Freeze-drying and milling. Extraction with (1) acetone/H_2O/acetic acid (70:29.5:0.5) and (2) acidic methanol (HCl)/H_2O (50:50 v/v, pH = 2.0, 1h, room temperature) followed by $(CH_3)_2CO$/H_2O (70:30 v/v, 1h, room temperature); Centrifugation.	Residue was hydrolysed with HCl/butanol (5:95) with $FeCl_3$ (100°C, 1h). Centrifugation Residue was treated with enzymes peptinase (37°C, 24h), esterase (55°C, 5h), protease (55°C, 5h) and a mixture (55°C, 5h)	Proanthocyanidin assay 555 nm (NEPAs). HPLC-fluorescence (silica column) (NEPAs).

Apples and apple peels (Malus domestica). Red and green grapes (*Vitis vinifera* L.). *Amelanchier alnifolia*, *Vaccinium vitisidaea* (berries). *Pinus sylvestris* [46]	Drying and milling. Extraction by sonication with (1) 80% aqueous acetone; (2) 70% acetone in aqueous 0.5% acetic acid; (3) acetone/methanol/water (2:2:1) (10min); Centrifugation; Evaporation; SPE DPA-6S 1 g polyamide cartridges; Freeze-drying	Thiolysis reaction	HPLC-DAD-MS (silica column) (NEPAs)
Blueberry leaves [47]	Drying and pulverization. Extraction with methanol/H_2O (50:50) (1h) followed by other extraction with acetone/H_2O (70:30) by sonication. Centrifugation and drying	Residue was hydrolysed with HCl/butanol (2.5: 97.5) with $FeCl_3$ (100°C, 1h). Centrifugation	Folin Ciocalteu (TPC), vanillin assay (PAs); 555nm (NEPAs)
Cranberry pomace [36]	Drying and grinding. The sample was treated with NaOH in a shaking water bath (200 rpm, 40 min, 60°C)	Residue was hydrolysed with NaOH (15 min at 60°C). Defatting with hexane. Successive extractions with ethyl acetate, neutralization, homogenization and filtration. Clean-up on SPE (Sephadex LH-20) column; Purification with alkaline treatment (NaOH, 15 min at 60°C)	HPLC-LC-MS (silica column) (NEPAs), MALDI-TOF/MS (NEPAs)
Malay cherry leaves *(Cepisanthes alata)* [38]	Freeze-drying and grinding. Extraction with water under agitation (200 rpm, room temperature, 12 h). Centrifugation and freeze-drying	Centrifugation and filtration (cellulose membrane). Thiolysis reaction	LC-ESI/MS, MALDI-TOF/MS (Develosil Diol column) (PPAs)
Ripe fruits quince, hawthorn, pear, blueberry [11]	Drying and pulverization. Boiled with ethanol (80%). Homogenization and filtration; Washing with ethanol (80), acetone (70%), ethanol (99.5%) and diethyl ether. Freeze-drying. Extraction with H_2O (16h)	The residue was extracted with CDTA (6h, 0.05M). The CDTA-insoluble residue was extracted with Na_2CO_3 solution (0.1M) containing 0.1% $NaBH_4$ (2°C, 20h and room temperature, 2h). Residue was treated with 4% KOH containing 0.1% $NaBH_4$ (18h, room temperature). Residue was extracted with 24% KOH containing 0.1% $NaBH_4$ (2h, room temperature) and treated with 4% KOH. Thiolysis reaction	HPLC-DAD (C18 column) (NEPAs)
Cauliflower byproducts (leaves) [48]	Grinding leaves with liquid N_2 and homogenisation using stirrer. Extractions with methanol. Vacuum-drying	Residue was hydrolysed with NaOH (60°C, 15 min) by sonication (37 kHz, 180W). Centrifugation; and clean-up on SPE (C-18) column. Drying and dilution in methanol	UHPLC-DAD (C18 column). UHPLC-ESI/MS (C18 column) (NEPs). Folin Ciocalteu (TPC)
Red cabbage and Brussels sprout waste streams [40]	Freeze-drying. Samples were diluted (100% methanol) and homogenised with ultraturrax. Centrifugation and re-extraction (80% methanol). Centrifugation. Drying	Residue was hydrolysed with NaOH by sonication (30 min, 60°C) and extracted with methanol (0.1% formic acid). Centrifugation and clean-up on SPE column (C-18)	UHPLC-ESI/MS (C18 column) (NEPs). Folin Ciocalteu (TPC).

Grape seed [34]	Air-drying (40°C). Immersion in liquid N_2 and grinding	Extraction with methanol/ H_2O (80:20) and with 75% acetone/H_2O (75:25) (3h, room temperature, each extraction). Extraction with hexane and reeze-drying. HSCCC. Preparative-HPLC; Phloroglucinolysis reaction	UHPLC-MS (C18 column) NMR (one dimensional) (PPAs)
Grape seed (*Malvar*) [49]	Grinding	UAE with methanol/H_2O (80:20, 15 min). Centrifugation and filtration with nylon filters. Freeze-drying	HILICxRP-LC-DAD-MS/MS (Monomers, oligomers and polymers of PAs)
Grape seeds and skins [50]	Freeze-drying and grinding	Enzymatic extraction by agitation (150 rpm, 3h) with pectinase (25°C), cellulose (37°C) and tannase (30°C). Solution in ethanol/ethyl acetate (50:50) and vortexed. Centrifugation and filtration. Phloroglucinolysis reaction	Folin-Ciocalteu (TPC), HPLC-UV (C18 column) (PPAs)
Persimmon [51]	Drying and cutting. Homogenization (ethanol 90%), filtration, evaporation and dilution in H_2O. Defatting with hexane and clean-up on SPE (HP-20) column	Residue was treated with 1.2 N HCl- 50% methanol solution (90°C, 3h). Centrifugation. Residue was treated with 1.2 N HCl- 50% methanol solution	Folin-Ciocalteu (TPC); Antioxidant capacity ORAC and DPPH (NEPs)
Persimmon pulp [52]	Inactivation polyphenol oxidase (H_20, 100°C, 10 min). Peeling and cutting with a stainless knike. Extraction with methanols acidified (1% HCl) (90°C, 30 min); Separation and purification: gluss column packed with AB-8 macroporous resin, ultrafine membrane	Samples were treated by thyolysis with 5% benzyl mercaptan in methanol containing 0.2M HCl (60°C,2h); Water and methanol solution was hydrolysed with 2 M HCl (80°C, 5h)	Folin-Ciocalteu (TPC); Vanillin assay (PAs); HPLC-ESI-MS (NEPAs).
Pomegranate [53]	Separation.	Extraction with acetone/water (70:30) and vacuum-drying. Clean-up on SPE (LH-20) column	MALDI-TOF/MS (HTs).
Pomegranate [42]	Washing and steaming. de-juiced (rack and cloth press); Drying.	Extraction with 80% methanol and 0.1% HCl	HPLC-ESI-MS (C18 column) (HTs)
Pomegranate juices, stems, leaves and flowers [54]	Juice was centrifuged and filtrated. Stems, leaves and flowers were freeze-dried and milled	Liquid samples were diluted and the solid samples were treated with methanol (magnetic stirring, 2h), centrifuged, filtrated and diluted (H_2O)	LC-ESI-TOF/MS (silica column) (HTs).
Pomegranate peel and seed (*Punica granatum* L), Grape seeds (*Vitis viniferaL.*), Myrtle leaves (*Myrtus communi*s L.) [41]	Freezing and crushing	Extraction with water (ebullition temperature) and concentration under vacuum. Washing (H_2O). Preparation of hydroalcoholic extracts with methanol/H_2O (70:30) and defatted with hexane. Concentration under vacuum	HPLC-DAD-ESI-MS (C18 column) (HTs).

Peanut hull skins [55]	Hulling and skinning, grinding and sieving. Defatting with hexane (Soxhlet) and drying. Regrinding and roasting at 150°C in an air draft oven for 30 min. Extraction with methanol/H_2O (50:50) and with acetone/H_2O (70:30) (1h, room temperature). Centrifugation	Residue was hydrolysed with HCl/butanol (40:50) (100°C, 3h). Centrifugation	555nm (NEPs)
Solid wastes from chestnut (peel) [56]	Peeling, freeze-drying and milling. Extraction with 1% Na_2SO_3/H_2O (960 min, 85°C) for HT and 1% NaOH in water during 240 min for PA. Filtration and freeze-drying	Residue was treated with methanol/H_2O (70:30, 70°C, 30min). Centrifugation	HPLC-DAD-MS (C18 column) (PAs and HTs).
Chick-pea, sweet chestnut, red lentils [7]	Milling and defatting with hexane and diethyl ether, 8h by Soxhlet. Extraction with methanol/H_2O (50:50) 3 min at room temperature followed by extraction with acetone/H_2O (70:30). Centrifugation	Residue was hydrolysed with methanol/H_2SO_4 (85°C, 20h). Washing and centrifugation	Folin-Ciocalteu (TPC). Antioxidant capacity FRAP (HTs).
Red and purple rice bran [57]	Sieving and flushing with N_2. Hexane extraction (2h, room temperature) followed by extraction with acetone/H_2O/acetic acid (70:29.5:0.5, 2h, room temperature). Centrifugation	Residue was hydrolysed with butanol/HCl and $FeCl_3$. Drying and clean-up on SPE (LH-20) column	DMAC assay (NEPAs). HPLC-fluorescence (Develosil diol column) (NEPAs).
Rice (*Oryza sativa* L.) [37]	Homogenisation in cutting mill. Extraction with 80% ethanol (10min). Centrifugation. Samples treatment with 80% ethanol and hexane (10min). Centrifugation	Residue was hydrolysed with NaOH (4M) acidified with HCl (6M) (room temperature, 4h); Centrifugation and extraction with ethyl acetate. Dried	Folin-Ciocalteu (TPC)
Wheat flour, wheat bran, white bread, rice, spaghetti, wheat breakfast cereals, croissant, biscuits. [17]	Drying and milling. Extraction with acidic methanol (HCl) and water (50:50, 1h, room temperature by agitation). Centrifugation. Extraction with acetone/ H_2O (70:30) and centrifugation	Residue was hydrolysed with methanol/H_2SO_4 (90:10, 85°C, 20h) and NaOH (2 M) with water (room temperature, 4h)	HPLC-DAD-MS (C18 column) (HTs)
Artichoke, cardoon, wild cardoon [58]	Grinding and sieving. Extraction with methanol/H_2O (50:50, 3 min at room temperature) followed by extraction with acetone/H_2O (70:30). Centrifugation	Residue was dried and hydrolysed with methanol/H_2SO_4 (85°C for 20h). Centrifugation, washing and centrifugation	Antioxidant capacity FRAP (HTs)
Honey [59]		Sample was solubilized in H_2O (agitation), filtrated and washed with ethanol/H_2O (50:50). A chromogenic reagent was added	KIO_3 assay (525nm) (HTs).
Averrhoa bilimbi (leaves and fruits). [39]	Freeze-drying and grinding. Extraction with acetone/H_2O (7:3 v/v) by magnetic stirring (1h). Filtration, centrifugation and freeze-drying	Thiolysis reaction	HPLC-MS (C18 column) (NEPAs)

Spatholobus suberectus [60]	Drying. UAE (acetone/H_2O, 70:30), evaporation and centrifugation. Extraction with hexane and partition with ethyl acetate. Freeze-drying and graded precipitation in different chloroform/methanol solutions. Centrifugation, and evaporation	Thiolysis reaction	ESI-QQQ/MS, MALDI-TOF/MS (PPAs)
Cinnamomi cortex [61]	Drying, pulverizing and sieving. MDDE with ethyl ether (35°C), dichloromethane (40°C), or petroleum ether (35°C). Centrifugation	Residue was transferred to around-bottom flask with ethanol/H_2O (85:15). Irradiation by microwave oven for 20 min. Filtration. Freeze-drying	GC-MS (Oligomeric and polymeric PAs). Vanillin assay (PPAs)
Uncaria tomentosa (leaves, stems, bark and wood) [62]	Drying at 40°C and grinding. Extraction of non-polar compound with a mixture of methyl tert-butyl ether and methanol (90:10, v/v) at 25°C during 30 min by UAE. Filtration and evaporation. Extraction with methanol at 25°C during 30 min by UAE. Filtration, washings and evaporation	Residue was treated with butanol/HCl (50:50) (0.54 mM $FeSO_4$) at 90°C during 1h	Proanthocyanidin assay (PAs). ESI-QTOF/MS (PAs)

CDTA, cyclohexane diamine tetraacetic acid; DAD, diode array detector; DMAC, dimethylaminoacinnamaldehyde; DPPH, α-diphenyl-β-picrylhydrazyl; ESI, electrospray ionization; EPP, extractable polyphenols; FRAP, ferric reducing ability of plasma; GC, gas chromatography; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; HPP, hydrolysable polyphenols; HSCCC, high-speed counter-current chromatography separation; HTs, hydrolysable tannins; MALDI, matrix-assisted laser desorption/ionization; MDDE, microwave-assisted simultaneous distillation and dual extraction; MS, mass spectrometry; NEPAs, non-extractable proanthocyanidins; NEPs, non-extractable polyphenols; ORAC, oxygen radical absorbance capacity; PAs, proanthocyanidins; PP, polymeric polyphenols; PPAs, polymeric proanthocyanidins; Q, quadrupole; QQQ, triple quadrupole; RP, reverse phase; SPE, solid phase extraction; TOF, time of flight; TPC, total phenolic content; UAE, ultrasound assisted extraction; UHPLC, ultra-high performance liquid chromatography.